



Can the Acid-formation Potential of Saliva Detect a Caries-related Shift in the Oral Microbiome?

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Purpose: To determine acid-formation potential of saliva and evaluate whether this method corresponds with microbiome composition of individuals with and without caries.

Materials and Methods: A clinical, controlled pilot study was performed with two groups: individuals without caries ($n = 25$; DMFT = 0) and individuals with at least one active carious lesion ($n = 25$; DMFT > 0). A detailed intraoral examination was performed, and the gingival bleeding index (GBI) and plaque index (PI) were recorded. The acid-formation potential was measured (ΔpH) after 1 h. *Streptococcus mutans* (SM) and lactobacilli (LB) were also quantified. Intergroup comparisons were made using the Mann-Whitney U-test. The diagnostic value was evaluated using the receiver operating characteristics (ROC) method and area under the curve (AUC) values were calculated. The saliva microbiome was analysed by 16S rDNA next-generation sequencing.

Results: A statistically significant difference was found in ΔpH , with the 'caries' group showing a higher mean value after 1 h ('healthy' = 1.1, 'caries' = 1.4; $p = 0.035$). The AUC values were moderate to good ($\Delta\text{pH} = 0.67$; SM = 0.83; LB = 0.83; 1 = ideal). *Streptococcus mutans* and Lactobacilli were more frequently detected in the 'caries' group ($p < 0.001$), as were statistically significantly higher GBI ($p = 0.006$) and PI ($p = 0.001$). The saliva microbiome had a higher α -diversity and greater richness in individuals with active caries. The incidence of the genera *Alloprevotella*, *Prevotella*, *Campylobacter* and *Veillonella* was statistically significantly higher in the 'healthy' group. The incidence of the genera *Fretibacterium*, *Lactobacillus*, and *Leptotrichia*, as well as the phyla Spirochaetes and Synergistetes, was statistically significantly higher in the 'caries' group.

Conclusion: Further studies must be carried out to determine the extent to which both tests are suitable for predicting future caries development.

Key words: acid formation, bacterial tests, caries, oral microbiome, saliva

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Determining a shift in an individual's oral microbiome from homeostasis to dysbiosis and therefore the increased risk of caries would be indispensable for planning preventive interventions in a timely manner so that irreversible loss of hard tooth substance can be prevented. Despite efforts to intervene early in the caries process, to date, the individual's

previous caries experience remains the most precise single parameter to predict caries risk.^{9,22,27,31,36,38,44}

Saliva has been used in commercially available tests and in clinical studies to determine the risk of caries.^{11,33,39} It can be extracted easily and non-invasively at low cost, and bacterial parameters can be obtained by quantifying caries-

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Table 1 Categorisation of acid-formation potential and corresponding caries risk as a function of pH difference (Δ pH) in a novel saliva test (patent pending)

Δ pH	Risk class	Acid-formation potential	Risk of caries
≤ 0.5	1	low	In combination with low bacterial count of MS and/or LB, no caries risk
0.5–1	2	medium	In combination with medium to high bacterial counts of MS and/or LB, increased caries risk
≥ 1	3	high	In combination with high bacterial counts of MS and/or LB, high caries risk
≥ 1.2	4	very high	Very high caries risk

MS: *Streptococcus mutans*; LB: lactobacilli.

associated species of mutans streptococci and lactobacilli. Furthermore, high-quality bacterial DNA can be extracted from saliva, making it an ideal substance for diagnostics. However, non-bacterial parameters, such as salivary flow rate, buffering capacity, and pH value, are only marginally suitable for predicting disease,^{3,19} and must be combined with other parameters, such as bacterial predictors. Although some studies have reported different microbial diversity between saliva and supragingival plaque,^{13,16,34} Shi et al³⁷ showed that the salivary microbiome has positive associations with the supragingival microbiome. This indicates that saliva samples may be useful for monitoring the supragingival microbiome. Indeed, a positive correlation was found between the numbers of mutans streptococci and lactobacilli in plaque and saliva.²³

Oral health is reflected in the balance between host and oral microorganisms. This describes the currently most widely accepted theory of the aetiology and pathogenesis of caries: the ecological plaque hypothesis.²¹ This hypothesis combines the specific and unspecific plaque hypotheses, and a shift in the described equilibrium (dysbiosis) enables the caries process. Continuous consumption of fermentable carbohydrates increases the number of acidogenic and aciduric microorganisms in saliva, which gradually displace non-pathogenic microbiota.⁴³ Consequently, these acidogenic and aciduric microorganism have the potential to change the environment in the oral cavity. The acid-formation potential of the saliva, which is measured after one hour of incubation, represents a new method that might be helpful in the detection of ecological and caries-related changes within the oral cavity.

This pilot study addresses the need for suitable test procedures and additional screening tools to intervene early in the caries process. As this is the first study set-up evaluating this new salivary parameter, we tested whether the acid-formation potential of saliva is able to discriminate between a 'caries' and 'healthy' group in accordance with data obtained from conventional bacterial tests and saliva microbiome analysis.

MATERIALS AND METHODS

This was an exploratory pilot study carried out as part of a clinical controlled cross-sectional investigation and was ap-

proved by the local medical ethics committee (S-389/2017). All patients gave written informed consent in accordance with the Declaration of Helsinki. Participants were selected for inclusion in the study based on the following inclusion and exclusion criteria:

Inclusion criteria

- Able to give consent
- Written informed consent
- Older than 18 years
- Participants with a DMFT value of 0 were allocated to the 'healthy' group
- Participants with a DMFT value of > 0 and at least on cavitated carious lesion were allocated to the 'caries' group.

Exclusion Criteria

Hyposalivation (unstimulated saliva flow rate below 0.1 ml/min and stimulated salivary flow rates below 0.7 ml/min) due to:

- Systemic diseases (Sjögren's syndrome, salivary gland diseases, diabetes mellitus, neurological diseases)
- Tumors, operations, and/or radiation in the head and neck area
- Psychological disorders
- Drugs with an anti-sialagogue effect (e.g. psychotropic drugs, appetite suppressants, antihypertensive agents, antihistamines, diuretics, cytostatics)
- Reduced chewing ability
- Use of antibiotics in the past 14 days
- Use of antibacterial mouthwash in the last 12 days
- Smoking or chewing gum within 2 h before the examination
- Eating or drinking directly before the examination.

Participants were divided into two groups ('caries' and 'healthy') according to the aforementioned criteria. In the 'healthy' group ($n = 25$), participants were naturally healthy (DMFT = 0) and had no prior history of caries or invasive restorative dental treatments. Participants with at least one cavitated caries (DMFT > 0) were allocated to the 'caries' group. The dental examination comprised an extraoral and intraoral examination, a sensitivity and percussion test, and systematic recording of the DMFT index. In accordance with Klein et al,¹⁷ we determined the DMFT index based on ex-

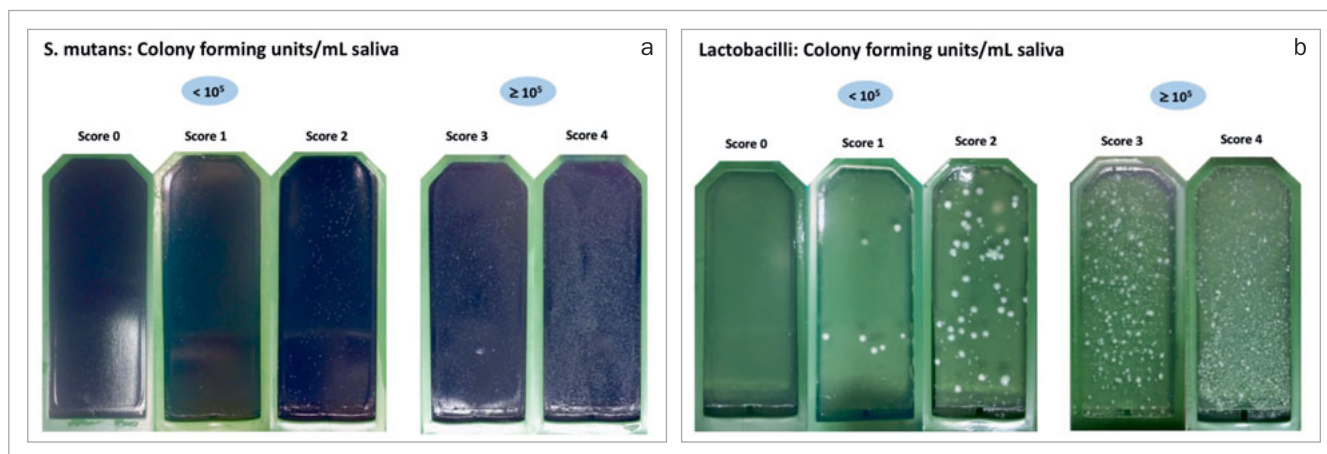


Fig 1 a. Classification of mutans streptococci CFU on a scale of 0–5 based on sample images from the present study. b. Classification of lactobacilli CFU on a scale of 0–5 based on sample images from the present study.

amination of 28 teeth. Any teeth with restorations or carious lesions were scored. Two probing depths (mesio- and distobuccal) were measured for each tooth and the bleeding-on-probing (BOP) index was recorded. The gingival bleeding index (GBI)² and the plaque index or plaque control record (PCR)²⁶ were also measured.

Saliva tests were performed on stimulated saliva (patients chewed paraffin). The following were measured for every participant:

1. the acid-formation potential of saliva after 1 h of incubation
2. the rate of secretion over 5 min
3. the buffering capacity
4. the number and occurrence of mutans streptococci and lactobacilli.

Acid-formation Potential

This novel saliva test determined the acid-formation potential or pH difference (Δ pH) of the saliva after 1 h of incubation (patent pending). The pH of the saliva was measured using McloropHast pH 6.5–10.0 pH indicator sticks (Merck; Darmstadt, Germany) before the test was carried out. One milliliter of the stimulated saliva was then pipetted into the test nutrient medium and incubated at 37°C for 1 h. Alternatively, the sample can be incubated for 1 h in the patient's pocket while professional tooth cleaning is carried out. After 1 h, the pH was measured using the special indicator McloropHast pH 5.2–7.2 (Merck), and the Δ pH was determined. Table 1 shows the acid-formation potentials and the corresponding risk of caries based on the Δ pH values measured in this new saliva test.

Bacterial Tests

The bacterial count was determined using the caries risk test (CRT, Ivoclar Vivadent; Schaan, Liechtenstein), a double-sided dip-slide agar carrier with selective culture media for mutans streptococci (Mitis salivarius bacitracin agar)

and lactobacilli (rogosa agar). After 48 h of incubation, colony-forming units (CFU) for mutans streptococci or lactobacilli were calculated per milliliter of saliva using a template supplied by the manufacturer. For a more precise evaluation, photographs were taken and sample images were assigned a score from 0 to 5 (Fig 1).

Buffering Capacity

The buffering capacity was measured using the CRT (Ivoclar Vivadent) and the Saliva-Check BUFFER test (GC Germany; Bad Homburg, Germany). Results were divided into low, medium, and high buffering capacity.

Saliva Microbiome

Before the dental examination, 2 ml of unstimulated saliva was taken from all participants and frozen at -80°C until microbiological analysis. The spitting method was used to collect unstimulated saliva that accumulated on the floor of the mouth and was spit into a pre-weighed test tube.²⁵ Bacterial DNA was extracted using the QIAamp DNA Mini Kit (Qiagen; Hilden, Germany) as specified by the manufacturer's instructions with partial modifications to the section on 'Isolation of genomic DNA from Gram-positive bacteria', 'Appendix D' (page 55), as previously described.⁵ Modifications consisted of using 180 μ l lysozyme (20 mg/ml) during the first lysis at 37°C for 30 min, then adding 20 μ l of proteinase K and 200 μ l of buffer AL and lysing only for 10 min without the step of 95°C for 15 min, to avoid DNA degradation. DNA quantity and quality were analysed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Germany; Braunschweig, Germany). The saliva microbiome was analysed using 16S rDNA next-generation sequencing. DNA was amplified using universal bacterial primers targeting the V4 region of the 16S rRNA gene (515F and 806R from Caspero et al⁶). Further descriptions of the microbiome sequencing technique are provided in Schoilew et al.³⁵

Table 2 Epidemiological and clinical data of study participants

Group		No. of samples	Sex		Age (years)	DMF(T)	D(T)	M(T)	F(T)
			female	male					
Group	Total	n = 50	48%	52%	28.8 ± 10.9 (18–65)	6.8 ± 8.1 (0–26)	2.0 ± 2.9 (0–11)	0.9 ± 2.2 (0–10)	3.9 ± 4.8 (0–15)
	Healthy	n = 25	44%	56%	24.5 ± 3.2 (19–31)	0.0 ± 0.0 (0–0)	0.0 ± 0.0 (0–0)	0.0 ± 0.0 (0–0)	0.0 ± 0.0 (0–0)
	Caries	n = 25	52%	48%	33.0 ± 13.9 (18–65)	13.6 ± 6.1 (1–26)	4.0 ± 3.0 (1–11)	1.8 ± 2.8 (0–10)	7.7 ± 4.1 (0–15)
Mann–Whitney U–Test (p)			0.575		0.01*				
DMF(T) values are presented as mean ± standard deviation (range).									

Table 3 Descriptive data of ΔpH values in the ‘healthy’ and ‘caries’ groups

Group		Participants	ΔpH M	ΔpH SD	ΔpH med	ΔpH Q1–Q3	ΔpH range
Caries	n = 25	1.4	0.6	1.4	0.9–1.9	0.5–2.5	
Mann-Whitney U-Test (p)		0.03536*					
M: mean; SD: standard deviation; med: median; Q1–Q3: interquartile range.							

Statistical Analysis

Data were analysed using descriptive and microbiological statistics. Descriptive analyses were performed using Software R, version 3.6.3³⁰ (R Foundation for Statistical Computing; Vienna, Austria) and included calculation of mean ± SD, range, medians and interquartile ranges. The Mann-Whitney U-test was carried out to determine statistically significant differences in ΔpH values, mutans streptococci and lactobacilli scores, GBI and PCR indices, and the buffering capacity between the groups. All p-values are purely descriptive. To evaluate the diagnostic quality of the test, receiver operating characteristic (ROC) curves were created and area under the curve (AUC) values were calculated.

Biostatistical analysis of the microbiological data and descriptive indices, such as alpha diversity (Shannon index), richness (number of ribosomal sequence variants [RSVs]), evenness (Pielou index), and dominance (Berger-Parker index), were carried out using the microbiome package. Permutational multivariate analysis of variance (PERMANOVA) was performed to determine statistically significant differences between the two groups. A DESeq2 analysis was also carried out to detect differences in RSV frequencies between the groups. Correlations between the microbiome indices, relative frequencies of RSVs, and quantitative clinical parameters were calculated using the Spearman correlation test, and p-values were adjusted using the Benjamini-Hochberg method. All microbiological analyses were carried out with R 3.1.4.²⁹

RESULTS

General Data

Table 2 shows the age, sex, and DMFT values of the two groups. Sex was fairly balanced (48% female, 52% male) and the mean age was 28.8 ± 10.9 years. Participants in the ‘caries’ group were statistically significantly older than those in the ‘healthy’ group (18–65 years vs 19–31 years; p = 0.01). In addition, there were statistically significant differences in GBI (p = 0.006) and PCR indices (p = 0.001) between the two groups, with higher values in the caries group. The mean GBI (%) value was 1.5 ± 2.9 in the ‘healthy’ group and 5.7 ± 6.8 in the ‘caries’ group. The mean PCR (%) was 28.4 ± 18.0 in the ‘healthy’ group and 46.4 ± 17.3 in the ‘caries’ group.

Acid-formation Potential

The saliva pH at the beginning of the test was not statistically significantly different between the two groups (‘healthy’ group, 7.6; caries group, 7.5). After 1 h, the ‘caries’ group had a statistically significantly higher mean ΔpH value than did the ‘healthy’ group (1.4 vs 1.1; p = 0.035) (Table 3, Fig 2). The ROC curve was only marginally better than a diagonal curve. The AUC (which indicates how well the acid-formation potential of saliva predicts the occurrence of caries) was 0.67 (Fig 3). Based on the ΔpH, acid-formation potential was rated as low, medium, high or very high (Table 1). Differences in acid-formation potential be-

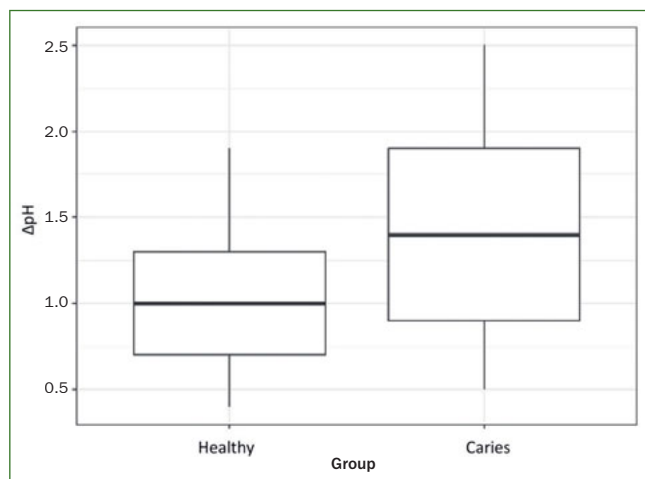


Fig 2 Comparison of ΔpH values (initial pH value minus pH value after 60 min of incubation in the nutrient medium of the new saliva test) between the 'healthy' ($n = 25$) and 'caries' ($n = 25$) groups. The 'caries' group had a statistically significantly higher ΔpH ($p = 0.035$).

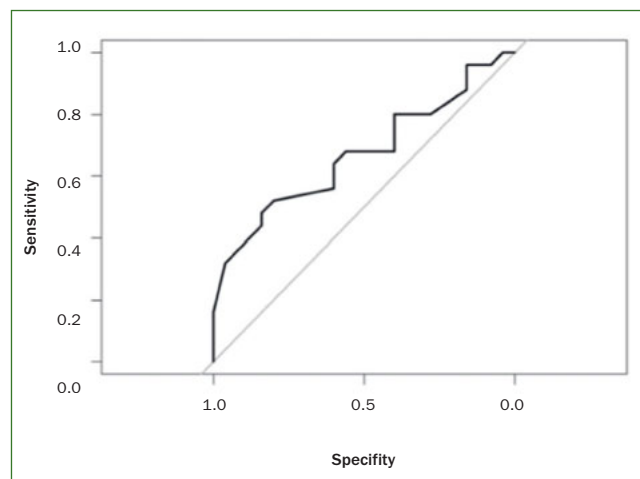


Fig 3 ROC curve of the measured ΔpH values of the 'healthy' ($n = 25$) and 'caries' ($n = 25$) groups. The AUC is the area under the ROC curve. The AUC value for ΔpH was 0.7 (1 = ideal).

tween groups are shown as percentages. These differences were greatest among participants with very high acid-formation potential, whereas 64% of the participants of the 'caries' group were attributed to that group (Table 4).

Bacterial Tests

Table 5 shows the results of the bacterial tests, scored from 0 to 4. No participants in the 'caries' group had a score of 0 or 1 for the distribution of mutans streptococcus, which indicates $< 10^5$ CFU. In contrast, 17 participants (68%) of the 'caries' group scored 4, while only 4 participants (16%) of the 'healthy' group scored 4. This difference was statistically significant ($p < 0.001$). In the 'healthy' group, 16 participants (64%) had a lactobacilli score of 0 and only 0–1 participants (0–4%) had a score of 3 or 4 (Table 5).

Buffering Capacity

The buffering capacity results are presented in Table 6. There were no statistically significant differences in buffering capacity between the two groups ($p = 0.905$).

Saliva Microbiome

A total of 954,209 'clean reads' (cleaned measured values) were obtained from the 50 samples and a mock community with an average of 19106 (8120–33353) sequences per sample. A total of 855 RSVs were found. There were an average 115.32 (74–195) RSVs per saliva sample. In the 'healthy' group, the abundance of the genus *Alloprevotella* as well as the RSVs belonging to the genera *Alloprevotella* (rsv104), *Prevotella* (rsv242), *Campylobacter* (rsv341), and *Veillonella* (rsv658) was higher. In the 'caries' group, there was a significantly higher abundance of the genera *Fretibacterium* and *Lactobacillus* and the phyla Spirochaetes and Synergistetes, as

well as the rsv816 belonging to the genus *Leptotrichia*. Figure 4 shows the most common RSVs of the saliva samples.

Figure 5 shows the structure of the saliva microbiome in the two groups according to principal coordinate analysis (PCoA) based on Morisita-Horn distances. Here, principal coordinate 1 represents 67.1% of the information and principal coordinate 2 represents 19.4%. Thus, the two principal coordinates together covered 86.5% of the total variance. Principal coordinate analysis revealed heterogeneity in both cohorts. Nevertheless, the samples of the 'healthy' group are separated from those of the 'caries' group, which is statistically validated by the PermaNova analysis (R^2 value = 0.131, $p = 0.003$).

Furthermore, the saliva microbiome was more diverse (Fig 6a) and richer (Fig 6b) in the 'caries' group than in the 'healthy' group.

DISCUSSION

In the present study, we showed that the acid-formation potential (ΔpH) of saliva was significantly higher in the 'caries' group than in the 'healthy' group. The AUC values, which indicate how suitable the acid-formation potential of saliva is for predicting a caries-related change in the oral microbiome, was moderate (Fig 3), and bacterial tests discriminated well.

These results are underlined by our finding that numbers of mutans streptococci and lactobacilli were statistically significantly higher in the 'caries' group (Table 5). The use of saliva to determine or screen individuals' caries risk remains controversial, and one-time saliva tests are known to be highly inaccurate, especially since other bacteria can be cultivated on the nutrient media. Because the results of one-

Table 4 Descriptive data of the acid-formation potential (Δ pH) of saliva in the ‘healthy’ group and the ‘caries’ group

		Participants	Acid-formation potential			
			Low	medium	high	very high
Δ pH			≤ 0.5	0.5–1	≥ 1	≥ 1.2
Group	Healthy	n = 25	8%	32%	20%	40%
	Caries	n = 25	4%	28%	4%	64%

Table 5 Descriptive bacteria test scores for mutans streptococci and lactobacilli counts in the ‘healthy’ group and the ‘caries’ group

		Participants	Score				
			0	1	2	3	4
Mutans streptococci counts							
Group	Healthy	n = 25	12%	20%	32%	20%	16%
	Caries	n = 25	0%	0%	12%	20%	68%
Mann-Whitney U-Test (p)		2.71e-05*					
		Participants	Score				
			0	1	2	3	4
Lactobacilli counts							
Group	Healthy	n = 25	64%	28%	4%	4%	0%
	Caries	n = 25	16%	20%	12%	28%	24%
Mann-Whitney U-Test (p)		2.374e-05*					

Table 6 Descriptive data for buffering capacity in the ‘healthy’ and ‘caries’ groups

		Participants	Low	Medium	High
Group	Healthy	n = 25	4%	44%	52%
	Caries	n = 25	16%	28%	56%
Mann-Whitney U-Test (p)		0.9047			

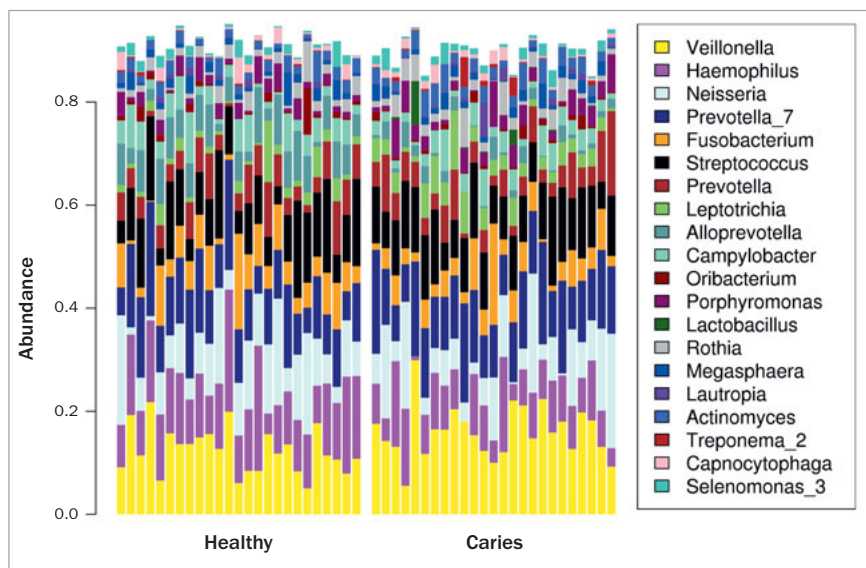
time tests are often left to chance, it is advisable to carry out repetitions to ensure the diagnostic value.^{10,12,32,36} However, we detected a positive correlation between the number of microorganisms in plaque and saliva, which agrees with published findings.^{23,37} This indicates that the microbiological composition of saliva may reflect the microbial composition of plaque.

This pilot study explored a new approach to assessing or screening risk of caries, starting with assessing intraoral acid-formation potential. Caries development is widely thought to be caused by a shift in the homeostasis of host bacteria and intraoral bacteria due to changes in local environmental conditions.²¹ These environmental changes may include acid production by any number of microorganisms.¹ The Δ pH values were statistically significantly higher in the

‘caries’ group after 1 h ($p = 0.035$, Table 3). In the ‘caries’ group, 64% of participants exhibited a very high acid-formation potential, supporting our earlier findings (Table 4). No differences in buffering capacity were detected between the two groups (Table 6), which corroborates published findings.³

Evaluation of the salivary microbiome revealed statistically significant differences in various genera and RSVs between the groups (Fig 4). Although Δ pH itself did not statistically significantly correlate to the microbiome, we were still able to detect a change in the microbiome between the two groups. Caries can be seen as a loss of protective function within the oral ecosystem, while the composition might be quite similar on the genus level. However, a few RSV and genera were statistically significantly different and the distance between the microbiomes moderate (R^2 value =

Fig 4 Bar chart of the 25 most common ribosomal sequence variants (genera) detected in saliva samples from the 'healthy' group (n = 25) and the 'caries' group (n = 25). The other genera were grouped together in the 'other' category.



0.131, $p = 0.003$), and an impact on the overall diversity and keystone genera was observed. In this context, the divergent acid-formation potential between the two groups might be potentially influence this change in the microbiome and therefore might contribute to the loss of function. Statistically significantly higher occurrences of *Lactobacillus*, *Fretibacterium*, *Spirochaetes*, *Synergistetes* and the RSV 816 (*Leptotrichia*) were detected in the 'caries' group. The higher incidence of lactobacilli is consistent with the CRT bacteria saliva test results described above (Table 5). Lactobacilli are not only marker bacteria,^{4,40} but are also predominantly associated with caries progression, as shown in previous studies.^{1,7,18,24} Thus, the results of the present study are consistent with previous reports. Our finding that *Leptotrichia* are more prevalent in the saliva microbiome of individuals with caries is consistent with the results of Ling et al²⁰ and Qudeimat et al.²⁸ In contrast, other studies have reported a higher occurrence of *Leptotrichia* in the saliva microbiome of individuals with no caries experience.^{1,35}

Two genera of the Prevotellaceae family are enriched in the salivary microbiome of the 'healthy' group (genus *Alloprevotella*, RSV104, and RSV 242). Both rsv242 (*Prevotella*) and rsv104 (*Alloprevotella*) had a statistically significantly higher occurrence in the 'healthy' group. It was observed that different *Prevotella* species were present in individuals with and without caries and that the species were not evenly distributed between these two groups. In a study by Schoilew et al,³⁵ *Prevotella* was one of 11 genera that occurred statistically significantly more frequently in individuals without caries experience compared to individuals with previous caries experience.

Another genus with a higher incidence in the 'healthy' group was *Veillonella* (RSV 658). According to Vesth et al,⁴¹ the importance of *Veillonella* in human infections is still uncertain, and this genus is generally considered to have low

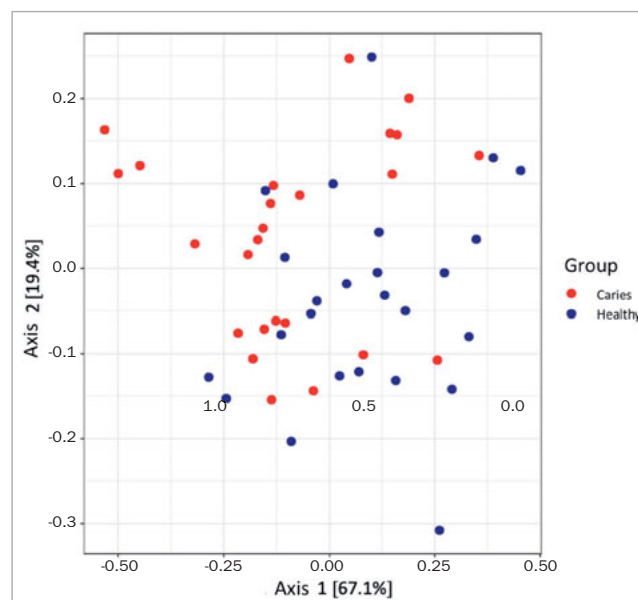


Fig 5 Comparison of the microbiome structure of the 'healthy' group (n = 25) and the 'caries' group (n = 25) using a principal component analysis based on Morisita-Horn distances. A heterogeneous distribution can be seen. 86.5% of the total variance is shown.

virulence. Nevertheless, *Veillonella* spp. have been associated with caries development.²⁸ Aas et al¹ showed that *Veillonella* spp. are present in intact enamel and carious lesions; however, *Veillonella* spp. are predominantly associated in stages within the carious process.

In this pilot study, α -diversity and richness were higher in the 'caries' group than in the 'healthy' group (Figs 5a and

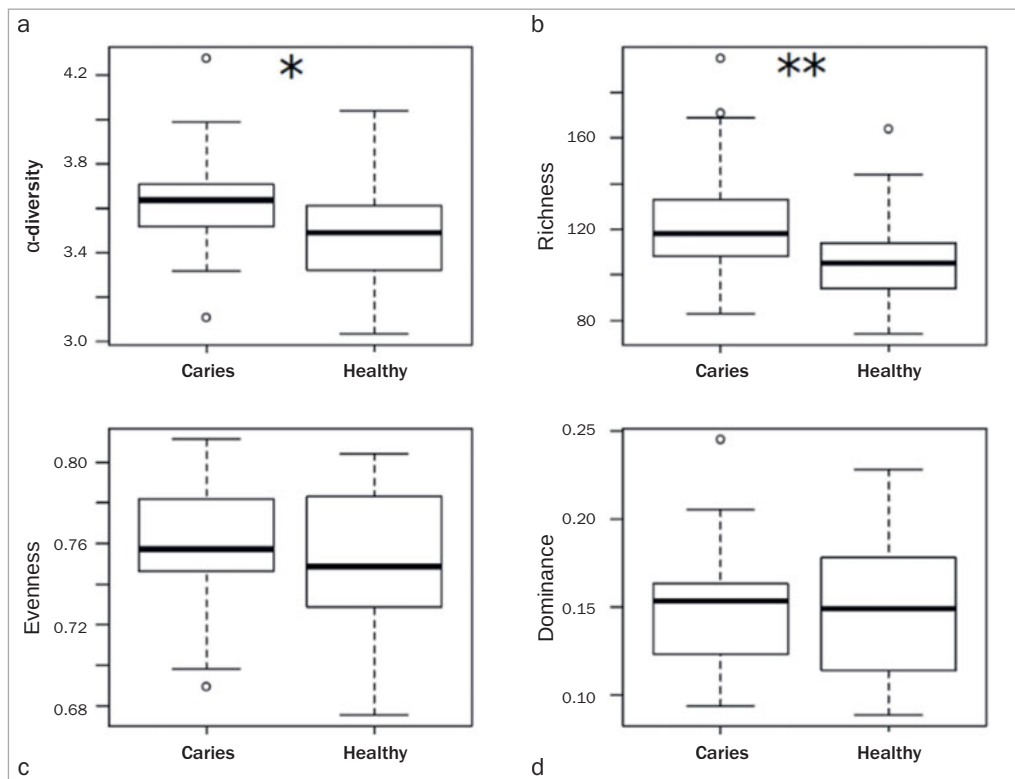


Fig 6 Box-and-whisker plots with medians and quartiles comparing the microbiome structure of the 'healthy' group (n = 25) and the 'caries' group (n = 25). a. Microbial α -diversity based on the Shannon index. b. Microbial richness, calculated as the number of observed RSVs. c. Microbial uniformity, based on the Pielou index. d. Microbial dominance, based on the Berger-Parker index. In (a) and (b), higher values were seen in the caries group. (c) and (d) are more balanced in the area of the median. However, the 'healthy' group shows a larger range of quartiles.

b), in agreement with previous findings by Yang et al.⁴² Earlier studies found a statistically significantly more variable microbiome in individuals with caries than in individuals without caries experience. In contrast, a recent study by Jiang et al¹⁴ showed no statistically significant differences in diversity and richness of the saliva microbiome in individuals with and without caries. It should also be noted that high diversity per se is not inevitably associated with a stable microbiome.^{8,15} Therefore, both diversity and stability of the microbiome are important for oral health.⁸

There are some limitations to the present study. The examinations were carried out by one dentist and the group sizes of 25 participants per group were quite small. Due to the preselection of the two groups, which was necessary to test the hypothesis, the assessment of the potential of Δ pH for risk prediction was not ideal. Therefore, the results should be interpreted with caution. Furthermore, saliva per se is subject to various intrinsic and extrinsic influences. For instance, the composition of saliva can be affected by biological fluctuations and circadian rhythms, particularly with regard to the microbiological composition. These influences may have affected our results. Besides, contradictory results from similar studies in that field may be related to variations in sampling, the degree of separation between and homogeneity within the cohorts being studied, as well as differences in DNA extraction and sequencing. Although current next-generation sequencing technology allows taxonomic profiling of microbiota at high resolution and depth, it

must nevertheless be taken into account that the taxonomic assignment with 16S rDNA to genus and species level may be a challenge. To assign species the amplicon sequence variant is currently the most precise method. However, the species annotation is depending on the 16S region sequence and its variability.

Finally, the present investigation is a cross-sectional study in which an association between salivary parameters and pre-existing caries could be shown. If the test is to be used for preventive purposes, a subsequent study must be carried out to determine the extent to which it is suitable for predicting future caries development. This can be done in a prospective longitudinal study by calculating the validity (sensitivity and specificity) of the test.

CONCLUSION

We showed that according to the differences found in the saliva microbiome, the acid-formation potential (Δ pH) of saliva was significantly higher in the 'caries' group than in the 'healthy' group. In terms of detecting a caries-related shift in the oral microbiome, overall, the acid-formation potential of saliva (Δ pH) discriminated to a moderate extent and bacterial tests discriminated well. Caries can be seen as a loss of protective function within the oral ecosystem, while the composition of the two groups might be quite similar on the genus level. We saw an impact on the overall diversity and a

keystone genus. In this context, the different acid-formation potentials between the two groups might be a factor potentially influencing this change in the microbiome and therefore might contribute to the loss of function. Further studies must be carried out to determine the extent to which the two tests are suitable for predicting future caries development.

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Conflict of interest statement

LL is the developer of the novel saliva test determining the acid-formation potential (patent pending). He was involved in the conception of the study and writing the manuscript. The study setup, ethical approval and all experiments, data assessment and analyses were performed independently by LSR, KS, AF SK, SB and CF without involvement of LL.

REFERENCES

- Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE, et al. Bacteria of dental caries in primary and permanent teeth in children and young adults. *J Clin Microbiol* 2008;46:1407–1417.
- Ainamo J, Bay I. Problems and proposals for recording gingivitis and plaque. *Int Dent J* 1975;25:229–235.
- Alaluusua S, Kleemola-Kujala E, Grönroos L, Evälahti M. Salivary caries-related tests as predictors of future caries increment in teenagers. A three-year longitudinal study. *Oral Microbiol Immunol* 1990;5:77–81.
- Beighton D. The complex oral microflora of high-risk individuals and groups and its role in the caries process. *Comm Dent Oral Epidemiol* 2005; 33:248–255.
- Boutin S, Hagenfeld D, Zimmermann H, El Sayed N, Hopker T, Geiser HK, et al. Clustering of subgingival microbiota reveals microbial disease ecotypes associated with clinical stages of periodontitis in a cross-sectional study. *Front Microbiol* 2017;8:340.
- Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal* 2012;6:1621–1624.
- Chhour K-L, Nadkarni MA, Byun R, Martin FE, Jacques NA, Hunter N. Molecular analysis of microbial diversity in advanced caries. *J Clin Microbiol* 2005;43:843–849.
- Coyte KZ, Schluter J, Foster KR. The ecology of the microbiome: networks, competition, and stability. *Science* 2015;350:663–666.
- Ewoldsen N, Koka S. There are no clearly superior methods for diagnosing, predicting, and noninvasively treating dental caries. *J Evid Based Dent Pract* 2010;10:16–17.
- Fontana M. The clinical, environmental, and behavioral factors that foster early childhood caries: evidence for caries risk assessment. *Pediatr Dent* 2015;37:217–225.
- Fontana M, Zero DT. Assessing patients' caries risk. *J Am Dent Assoc* 2006;137:1231–1239.
- Hausen H. Caries prediction—state of the art. *Comm Dent Oral Epidemiol* 1997;25:87–96.
- Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, et al. Structure, function and diversity of the healthy human microbiome. *Nature* 2012;486:207.
- Jiang Q, Liu J, Chen L, Gan N, Yang D. The oral microbiome in the elderly with dental caries and health. *Frontiers in cellular and infection microbiology* 2019;8:442.
- Johnson KV-A, Burnet PW. Microbiome: Should we diversify from diversity? *Gut Microbes* 2016;7:455–458.
- Keijsers B, Zaura E, Huse S, Van der Vossen J, Schuren F, Montijn R, et al. Pyrosequencing analysis of the oral microflora of healthy adults. *J Dent Res* 2008;87:1016–1020.
- Klein H, Palmer CE, Knutson JW. Studies on dental caries: I. Dental status and dental needs of elementary school children. *Public Health Reports* (1896–1970) 1938:751–765.
- Lapirattanakul J, Nomura R, Okawa R, Morimoto S, Tantivitayakul P, Maudcheingka T, et al. Oral lactobacilli related to caries status of children with primary dentition. *Caries Res* 2020;54:1–11.
- Leone CW, Oppenheim FG. Physical and chemical aspects of saliva as indicators of risk for dental caries in humans. *J Dent Educ* 2001;65:1054–1062.
- Ling Z, Kong J, Jia P, Wei C, Wang Y, Pan Z, et al. Analysis of oral microbiota in children with dental caries by PCR-DGGE and barcoded pyrosequencing. *Microbiol Ecol* 2010;60:677–690.
- Marsh PD. Microbial ecology of dental plaque and its significance in health and disease. *Adv Dent Res* 1994;8:263–271.
- Mejäre I, Axelsson S, Dahlén G, Espelid I, Norlund A, Tranæus S, et al. Caries risk assessment. A systematic review. *Acta Odontol Scand* 2014;72:81–91.
- Mundorff SA, Eisenberg AD, Leverett DH, Espeland MA, Proskin HM. Correlations between Numbers of Microflora in Plaque and Saliva. *Caries Res* 1990;24:312–317.
- Munson M, Banerjee A, Watson T, Wade W. Molecular analysis of the microflora associated with dental caries. *J Clin Microbiol* 2004;42:3023–3029.
- Navazesh M. Methods for collecting saliva. *Ann N Y Acad Sci* 1993;694: 72–77.
- O'Leary TJ, Drake RB, Naylor JE. The plaque control record. *J Periodontol* 1972;43:38–38.
- Powell LV. Caries prediction: a review of the literature. *Comm Dent Oral Epidemiol* 1998;26:361–371.
- Qudeimat MA, Alyahya A, Karched M, Behbehani J, Salako NO. Dental plaque microbiota profiles of children with caries-free and caries-active dentition. *J Dent* 2020:103539.
- R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing. R Foundation for Statistical Computing, Vienna: R Foundation for Statistical Computing, 2014.
- R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing Vienna, Austria, 2020.
- Raitio M, Pienihäkkinen K, Scheinin A. Assessment of single risk indicators in relation to caries increment in adolescents. *Acta Odontol Scand* 1996;54:113–117.
- Reich E, Bößmann K. Stellenwert der Speicheldiagnostik im Rahmen der Kariesprävention. *Wissenschaftliche Stellungnahme der DGZMK* 1994; 4:94.
- Reich E, Lussi A, Newbrun E. Caries-risk assessment. *Int Dent J* 1999; 49:15–26.
- Ren W, Zhang Q, Liu X, Zheng S, Ma L, Chen F, et al. Exploring the oral microflora of preschool children. *J Microbiol* 2017;55:531–537.
- Schoilew K, Ueffing H, Dalpke A, Wolff B, Frese C, Wolff D, et al. Bacterial biofilm composition in healthy subjects with and without caries experience. *J Oral Microbiol* 2019;11:1633194.
- Schwendicke F. Baseline caries prevalence was the most accurate single predictor of caries risk in all age groups. *Evid Based Dent* 2013;14: 102–102.
- Shi W, Tian J, Xu H, Zhou Q, Qin M. Distinctions and associations between the microbiota of saliva and supragingival plaque of permanent and deciduous teeth. *PLoS one* 2018;13:e0200337.
- Twetman S, Fontana M. Patient caries risk assessment. In: Pitts N (ed). *Detection, assessment, diagnosis and monitoring of caries*. Basel: Karger Publishers, 2009:91–101.
- Van Houte J. Microbiological predictors of caries risk. *Adv Dent Res* 1993;7:87–96.
- Van Houte J. Role of micro-organisms in caries etiology. *J Dent Res* 1994;73:672–681.
- Vesth T, Ozen A, Andersen SC, Kaas RS, Lukjancenko O, Bohlin J, et al. Veillonella, Firmicutes: microbes disguised as Gram negatives. *Stand Gen Sci* 2013;9:431–448.
- Yang F, Zeng X, Ning K, Liu K-L, Lo C-C, Wang W, et al. Saliva microbiomes distinguish caries-active from healthy human populations. *The ISME journal* 2012;6:1–10.
- Zero D. Sugars—the arch criminal? *Caries Res* 2004;38:277–285.
- Zero D, Fontana M, Lennon ÁM. Clinical applications and outcomes of using indicators of risk in caries management. *J Dent Educ* 2001;65: 1126–1132.

